

# MOLECULAR AND MICROBIOLOGICAL APPROACHES FOR RAPID ETIOLOGICAL DIAGNOSIS OF SYSTEMIC MYCOSES

## Review

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## ABSTRACT

The review presents common laboratory methods for the diagnosis of the majority of invasive fungal diseases, i. e. candidosis, aspergillosis and cryptococcosis. Some studies reveal an increasing range of the infectious agents such as *Trichosporon* and endemic fungal pathogens like *Histoplasma*, *Blastomyces* and *Coccidioides*.

The most common serological tests for antigen and antibody-detection in body fluids (serum, plasma, bronchoalveolar lavage) are: indirect immunofluorescence, ELISA – Platelia (enzyme linked immunosorbent assay), latex-agglutination, immunodiffusion and molecular techniques like PCR and Real Time PCR.

Not only current data on invasive fungal disease diagnostic methods are reviewed, but also studies on new biomarkers and recent discoveries in molecular diagnostics.

New molecular approaches are needed to provide faster results.

**Key words:** *systemic fungal disease; Candida, Aspergillus, Cryptococcus, Real Time PCR*

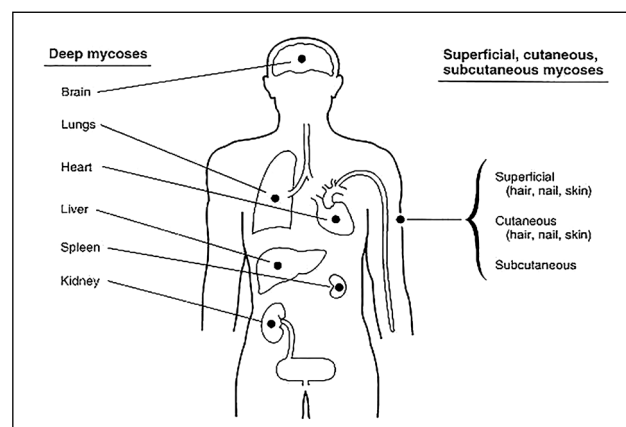
## INTRODUCTION

It is estimated that around 1.9 million patients worldwide develop invasive fungal disease (IFD) each year. These infections are associated with high mortality and economic burden due to hospitalizations. Therefore, a fast and timely diagnosis is needed (1).

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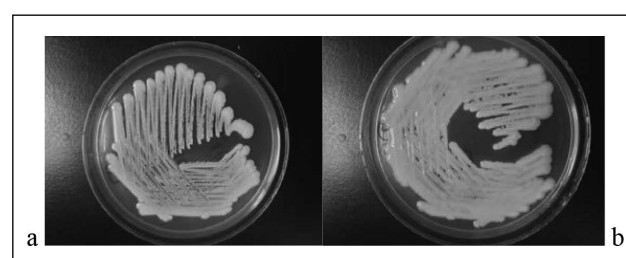
Systemic fungal diseases may cause mortality in immunocompromised individuals (oncology, chemotherapy, transplantation, catheterized or heart prosthetic and AIDS patients, patients in intensive care units (ICU), etc.) (fig.1).



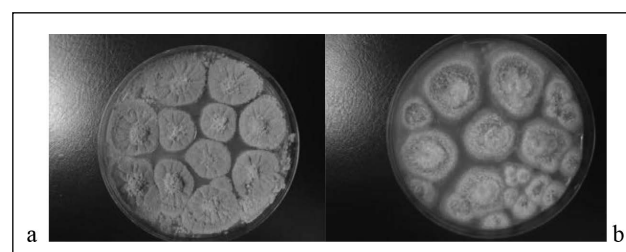
**Fig.1** Schematic representation of the types of fungal infections (<https://microdok.com/>)

Routine microscopic and culture based methods are the classical ones, but diagnosis is provided in 48-72 hours (2, 3) (fig.2, 3). For this reason, faster and reliable at the same time screening methods for early detection of pathogenic fungi are needed. (4, 5).

The majority of invasive fungal diseases are still caused by *Candida* (6) and *Aspergillus* species, but recent studies indicate an increasing incidence of other species, such as *Cryptococcus* and *Trichosporon* (7). There are endemic fungi in some areas, for example in the USA *Histoplasma*, *Blastomyces* and *Coccidioides* are reported (8).



**Fig.2.** Macroscopic view of *Candida albicans* (a) and *Cryptococcus neoformans* (b)-



**Fig.3.** Macroscopic view of *Aspergillus flavus* (a) and *Aspergillus fumigatus* (b)

Microbiological laboratory diagnostics most often includes:

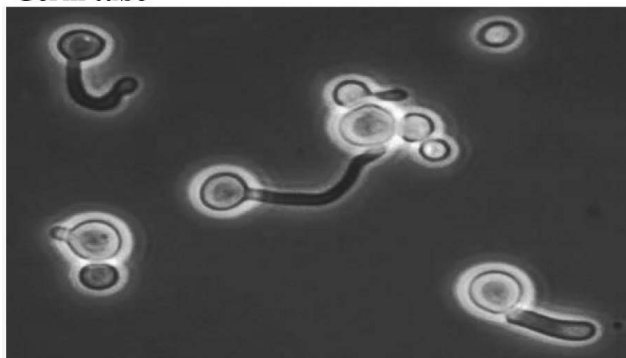
- Microscopy - directly from the clinical specimen or after an appropriate staining, by Gram, for example;
- Histology - evidence of fungal elements from biopsy;
- Culture examination - on culture media, including blood cultures, followed by identification and test for sensitivity to antifungals (antimycogram);
- Serology - detects antigens or specific antibodies;
- Molecular genetic methods, i.e. PCR;

### MICROSCOPIC EXAMINATION

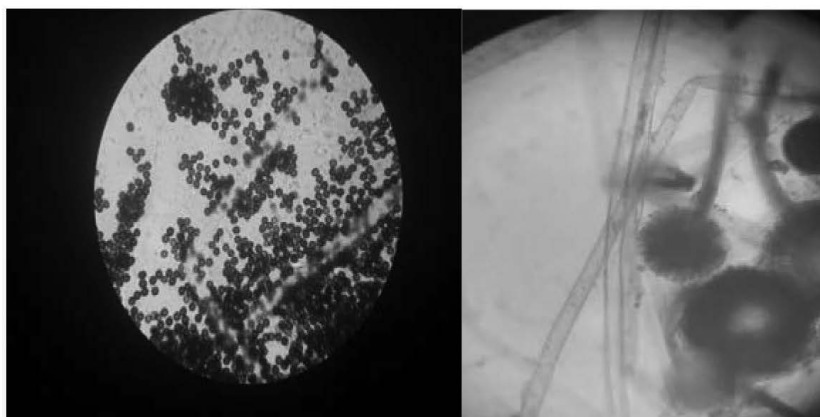
Microscopy of fungi can be done following appropriate staining - by Gram, or by fluorescent dyes, cotton blue, etc.

The formation of germ tubes in *C. albicans* is a factor of virulence, which can be detected by the so-called Germination test (Fig 4).

**Germ tube**



**Figure 4.** *Candida albicans* and a central true germ tube ([www.mycology.adelaide.edu.au](http://www.mycology.adelaide.edu.au))



**Fig.5.** Microscopic view and schematic representation of the microscopic characteristics in *Aspergillus* ([www.mycology.adelaide.edu.au](http://www.mycology.adelaide.edu.au))

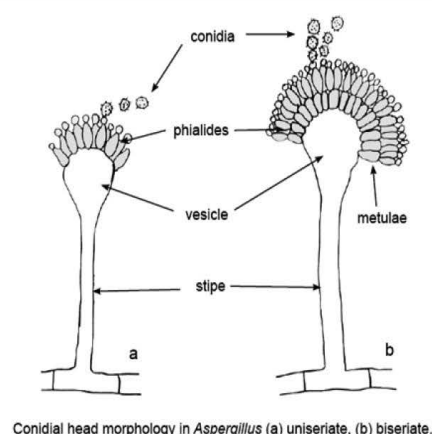
Microscopy and identification of *Aspergillus* is not easy and requires a guidance. *Aspergillus* spores are carried by a long conidiophore, and it originates from the so-called „foot cell“ (Fig.5). On its top it ends with the so-called vesicle, which can vary in shape and size. From the surface of the vesicle conidia-forming cells called metulae and phialides, begin, which can partially or completely cover the vesicle.

### HISTOLOGY

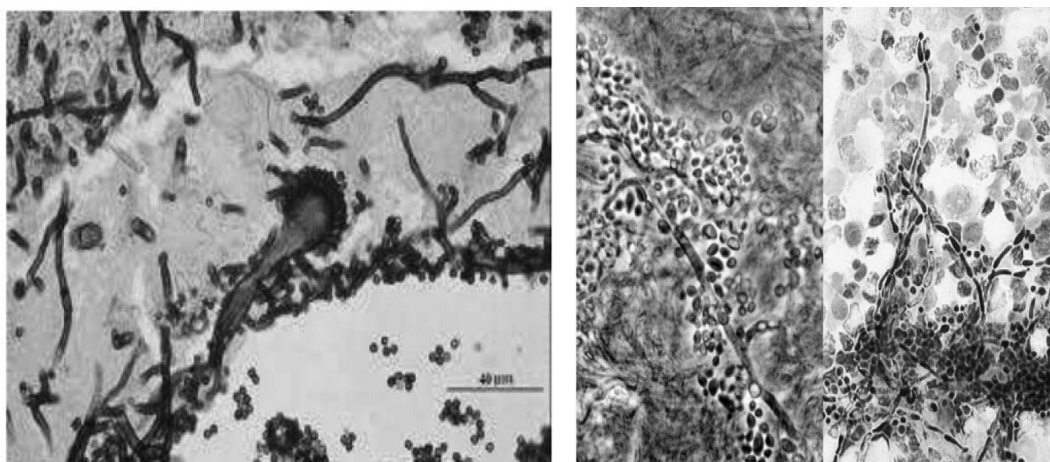
Histological examination of biopsy materials for the presence of fungal elements is considered one of the main criteria for a proven fungal infection (Fig 6). Biopsy or surgical resection of pathological tissue are often definite signs for the diagnosis of an IFD). Timeliness and expedience are essential for early diagnosis.

### CULTURE EXAMINATION

Sabouraud dextrose agar or chromogenic medium with color reaction during growth for 48-72 hours at 30°C, the so called Chrom agar, is the most often used solid nutrient medium for the culture study. Following isolation of a fungal strain as a pure culture, biochemical identification follows. This can be achieved with the help of non-automated (commercial identification kits) or automated identification systems (VITEK2, Merlin, Phoenix, MALDI-Toff, Microscan, etc.). There are various biochemical identification kits, the most commonly used of which are Api 20 CAux and Auxacolor.



Conidial head morphology in *Aspergillus* (a) uniseriate, (b) biserial.

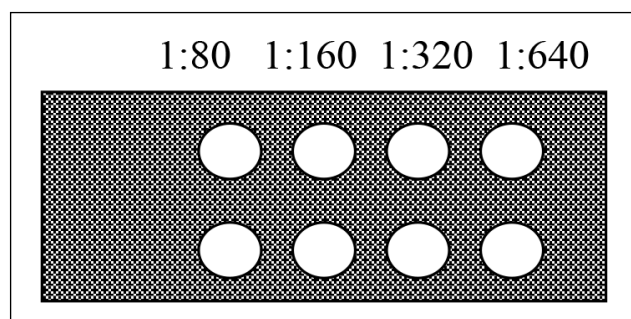


**Fig .6.**  
Visualization  
of fungal  
elements in  
biopsy tissue  
([www.mycology.adelaide.edu.au](http://www.mycology.adelaide.edu.au))

### ANTIBODY DETECTION

The method that we use for determination of specific antibodies against *Candida*, *Aspergillus* and *Cryptococcus* is the indirect immunofluorescence (IIF). For performing it, it is necessary to prepare a microscopic slide from a culture suspension ( $10^6$ - $10^7$  CFU/mL) of *Candida* strain (CIP 628), *Aspergillus* (*A. fumigatus*) and *Cryptococcus neoformans*. The slides with the fixed suspension should be stored at  $-20^{\circ}\text{C}$  (9).

The principle of the method includes 2 steps: in the first step, cover glasses should be coated with diluted serum samples (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1: 640 for *Candida* and *Aspergillus* or 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 – for *Cryptococcus neoformans*) for 30 minutes. In the second step, after washing the slides, the attached antibodies are stained with fluorescein-labeled anti-human immunoglobulin antibodies and visualized under the fluorescent microscope (fig.7) (10;11; 12).



**Fig.7.** IIF for the detection of serum antibodies.

### ANTIGEN DETECTION

In our laboratory we have experience about serology antigen detection with enzyme linked

immunosorbent assay (ELISA).

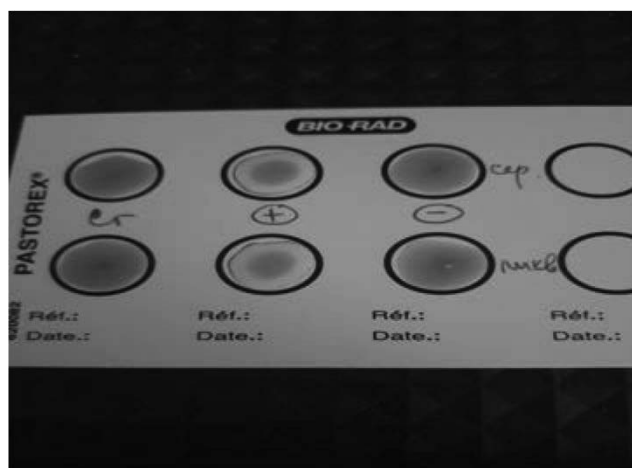
ELISA – Platelia (BioRad) is an immunoenzyme sandwich microplate assay for detection of the circulating antigen (mannan in *Candida* or galactomannan in *Aspergillus*) in human serum and in bronchoalveolar lavage (BAL) fluid (fig.8). Mannan and galactomannan antigen are polysaccharides non-covalently bound to the yeast cell wall and they appear to be the main biomarkers for the diagnosis of IFD.



**Fig.8** Elisa - Platelia commercial kits (BioRad, France) and schematic presentation of the sandwich Elisa method ([www.microbionotes.com](http://www.microbionotes.com))

Another serological test for the detection of circulating antigens using a sample technique, is the latex agglutination (LA) test (BioRad). It is a qualitative test detecting the polysaccharide antigen, mannan (for *Candida*) and glucuronoxylomannan (*Cryptococcus neoformans*) in biological fluids (serum, BAL, cerebro-spinal fluid (CSF)). The procedure uses latex particles coated with monoclonal rat antibody directed against the fungal antigen (13). The particles react with the fungal antigen resulting in agglutination visible to the naked

eye (fig.9) (14). Detection of *Candida* antigen by LA in body fluids is a reliable criterion for a systemic infection. LA tests for antigen detection in immunocompromised patients with cryptococcosis of the central nervous system (CNS) are even more reliable. The test has a very high sensitivity and specificity.



**Fig. 9.** LA test for yeast

A third method for antibody detection is immunodiffusion (ID). In our laboratory we have experience for searching antibodies against *Aspergillus* and endemic fungal pathogens such as *Histoplasma*, *Blastomyces* and *Coccidioides* – the spill phenol agar for ID in a few petri plates. Immediately after spilling agar, the petri plate is placed in the refrigerator at 4°C. After making wells with smooth edges, we drip:

- Control serum (commercial kit)
- Antigen (commercial kit)
- Patient's serum

Precipitation lines are sought following incubation in a wet camera at 25°C for a few days (9).

### MOLECULAR GENETIC METHODS FOR DIAGNOSIS

The non-culture based method is PCR – DNA-detection for the rapid diagnosis of infections – amplification of a specific DNA-fragment by means of short, artificially synthesized oligonucleotides (primers) (15;16). They hybridize specifically with a particular region of the target DNA, with participation of Taq-polymerase (9).

The following are the three reaction steps in one PCR cycle:

1. Denaturation of double-stranded DNA (dsDNA) to two single-stranded chains at 95°C (also called DNA melting)
2. Binding of primers to their complementary regions in the single-stranded ssDNA chains (also called annealing of primers)
3. Amplification of new DNA at each end in the 5'-3' direction, using DNA Taq polymerase in the presence of deoxynucleotides (dATP, dTTP, dCTP and dGTP) to build new daughter DNA fragments.

### REAL-TIME PCR

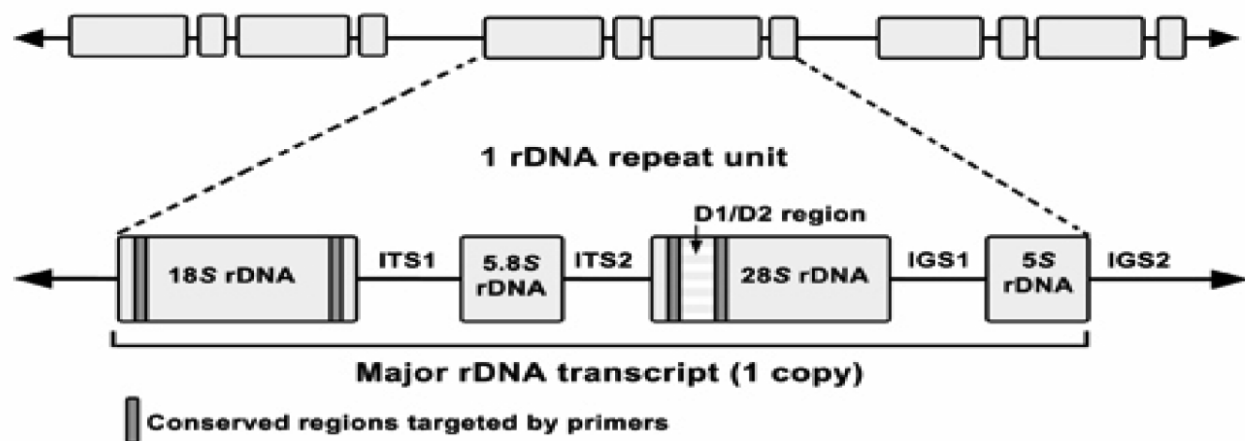
Real-time PCR allows tracking of DNA amplification at the time it occurs, not at the end of the reaction, as in conventional PCR (17).

In most eukaryotes, coding rDNA genes and spacers are found in tandem repeats separated by non-transcribable DNA, called "no"-transcribed spacer (NTS). The coding regions of 18S, 5.8S and 28S nuclear ribosomal DNA (rDNA genes) are relatively conserved among fungi and provide a molecular basis for establishing phylogenetic links. Between the coding regions are: the internal transcribed spacer 1 and 2 - regions, respectively ITS1 and ITS 2. The ITS region is perhaps the most widely sequenced DNA region in fungi. Universal fungal primers are used to amplify the entire internal transcribable ITS spacer. The ITS region, located between 18 S and 28S ribosomal DNA, is divided into two (ITS 1 and ITS2), and between them the preserved region 5.8S is situated (Fig.10)

Amplification techniques offer increased sensitivity over traditional staining and culture methods but may report positive results in asymptomatic individuals because of colonization (18; 19).

Real time PCR has advantages as speed, enhanced sensitivity and wider range of detectable organisms, which cannot be cultured (20).

Double-labeled hybridization probes can be used. The probe is an oligonucleotide that is labeled with a fluorescent dye (reporter) at one end and a fluorescence quencher at the other end. The proximity of the extinguisher to the fluorescent dye suppresses the emission of the natural fluorescence by the fluorescent dye. In

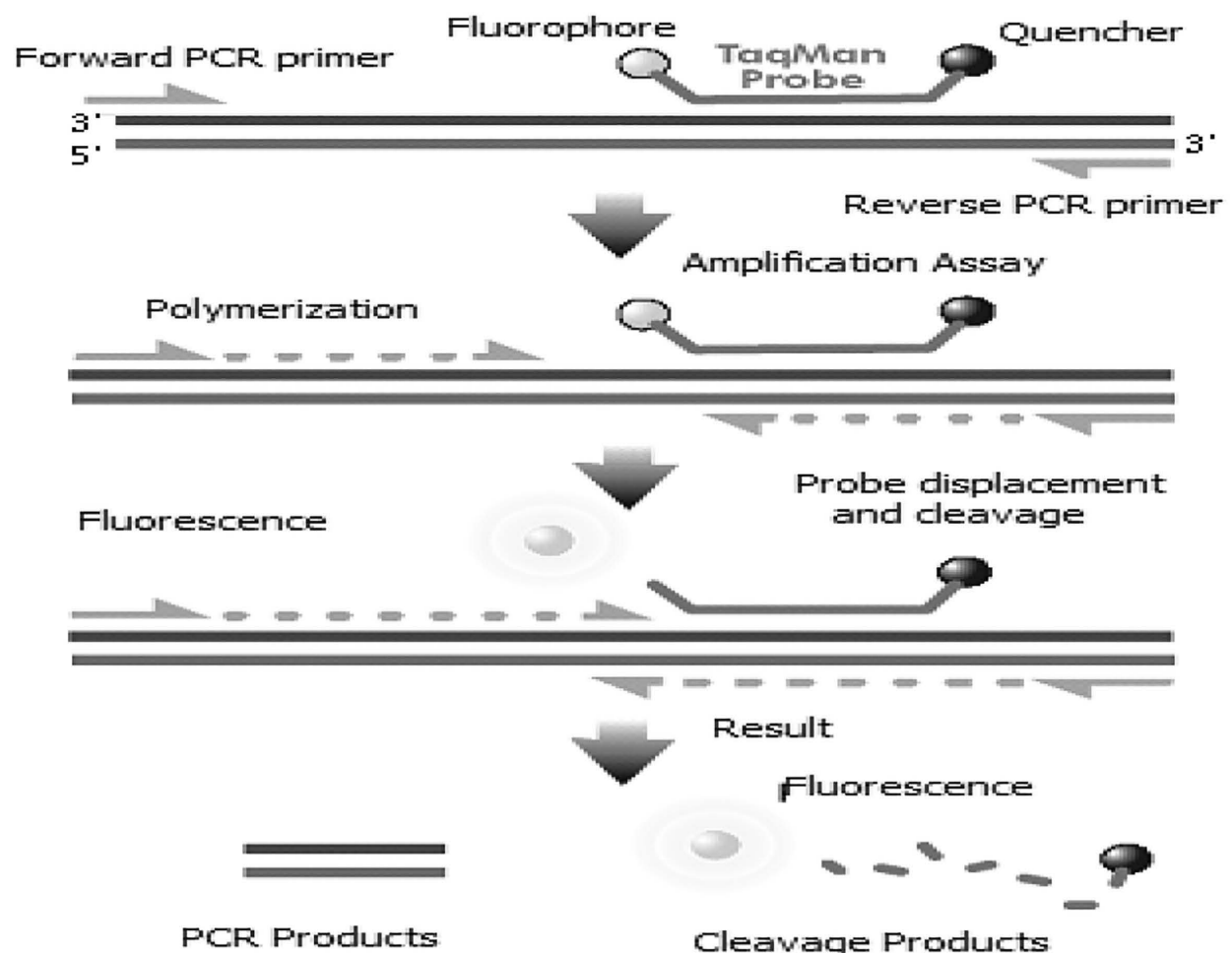


**Fig.10** Schematic representation of the organization of an rDNA-transcribed region comprising 18S, 5.8S and 28S-genes in fungi. Between the genes for 18 S and 5.8S rRNA is ITS1, and between the genes for 5.8 S and 28 S rRNA is ITS 2 ([www.researchgate.net](http://www.researchgate.net), [www.mycology.adelaide.edu.au](http://www.mycology.adelaide.edu.au))

PCR, the hybridization probe binds to a specific region of the template DNA. Thus, there is a directly proportional correlation between the increased fluorescent signal and the amount of the PCR product (Fig.11)

Real-Time PCR can also be used for quantitative

analysis - determination of the number of DNA molecules in the sample in real time - to distinguish colonization from infection, because some medically important fungi are opportunistic pathogens. They are part of the normal human microflora.



**Fig. 11.**Real-Time PCR (<https://www.wikiwand.com>)

The treatment options for invasive fungal infections are presented in a table 1.

**Table 1.** Recommendations for therapy of SANFORD guide, USA

| <b>SANFORD</b>                     | <b>Primary therapy</b>   |   | <b>An alternative</b>  |   |
|------------------------------------|--|---|--|---|
| <b>Invasive aspergilosis</b>       | <b>-Voriconazole</b> 6 mg/kg iv/po q 12h on day1, then 4 mg/kg iv/po q12h or<br><b>-Isavuconazole</b> 372 mg po/iv q 8hx6 doses, then 372 mg po/iv daily   |   | <b>-Liposomal Amphotericin B(L-AmpB)</b> 3-5 mg/kg/day iv or<br><b>-Amphotericin B lipid complex(ABLC)</b> 5 mg/kg/day/iv or<br><b>-Posaconazole</b><br>-300 mg po 2 bid x2 doses, then 300mg po/day or<br>-suspension 200 mg qid, then 400mg po bid or<br>- iv 300mg over 90 min bid/day 1, после 300mg iv/day  |   |
| <b>Invasive Candidosis</b>         | <b>Empirical therapy</b>   | <b>Target therapy</b>   | <b>Patients without neutropenia</b>  | <b>Patients with neutropenia</b>  |
|                                    | <b>-Caspofungin</b> 70mg iv, then 50 mg iv qd or<br><b>-Micafungin</b> 100 mg iv qd or<br><b>-Anidulafungin</b> 200 mg iv, then 100 mg iv qd   | <b>-for <i>C.albicans</i>, <i>C.parapsilosis</i>, <i>C.tropicalis</i>: Flucanazole</b> 800 mg(12 mg/kg), then 400 mg iv/po qd o pure blood culture and clinical stability<br><b>-for <i>C.glabtara</i>: echinocandins or Voriconazole</b><br><b>-for <i>C.krusei</i>: echinocandins,</b> then <b>Voriconazole</b> 6mg/kg bidx 2 doses, then 4 mg/kg bid o pure blood culture and clinical stability | <b>-Lipid-based Amphotericin B</b> 3-5 mg/kg iv qd<br><b>- Amphotericin B</b> 0.7mg/kg iv qd<br><b>-Voriconazole</b> 6mg/kg bidx2 doses,then 4 mg/kg bid   | <b>- Lipid-based Amphotericin B</b> 3-5 mg/kg iv qd<br><b>- Amphotericin B</b> 0.7mg/kg iv qd<br><b>- Voriconazole</b> 6mg/kg bidx2 doses, then 4 mg/kg bid<br><b>-Fluconazole</b> 800mg(12mg/kg), then 400 mg iv/po qd |
| <b>Cryptococcosis (meningitis)</b> | <b>- Liposomal Amphotericin B(L-AmpB)</b> 3-4 mg/kg iv q24h or<br><b>- Amphotericin B lipid complex(ABLC)</b> 5 mg/kg iv q 24h+Fluconazole 25 mg/kg po q6h<br>- can with <b>:Fluconazole</b> 400-800 mg po/day/8 weeks |   | <b>- Liposomal Amphotericin B(L-AmpB)</b> 3-4 mg/kg iv q24h or <b>Amphotericin B lipid complex</b> 5 mg/kg iv q 24h or <b>Amphotericin B</b> 0.7-1 mg/kg iv q24h+ <b>Fluconazole</b> 800-1200mg/day iv/po/2weeks<br><b>- Liposomal Amphotericin B</b> 3-4 mg/kg iv q24h или <b>Amphotericin B lipid complex</b> 5 mg/kg iv q 24h or <b>Amphotericin B</b> 0.7-1 mg/kg iv q24h/4-6 weeks<br><b>-Fluconazole</b> 800-1200/day iv/po+ <b>Flucytosine</b> 25 mg/kg po q6h/4-6 weeks<br><b>-Fluconazole</b> 1200-2000 mg po/day/10-12 weeks |   |

Abbreviations: iv – intravenous; po – per os; qd (quaque dia) – once at the appointed time; bid – twice a day

## GENERAL CONSIDERATIONS

The different methods for laboratory diagnosis of IFD vary in specificity and sensitivity (21).

For *Candida* spp, there are now direct tests to detect DNA. Recently, the Food and Drug Administration (FDA) approved a test with direct detection in blood samples of DNA of the most commonly isolated *Candida* species, with high sensitivity and specificity (95%) (T2 *Candida*; T2 Biosystems, Inc, Lexington, MA) (1).

New biomarkers for invasive aspergillosis (IA) – studies are being performed to detect volatile organic compounds in exhaled air as a result of *Aspergillus* metabolism (respiratory samples of patients with IA and those without IA are compared). Other studies have focused on the search for pan-fungal serum disaccharide (DS) as an additional marker in the diagnosis of fungi (1). Antibody titers to antigens of the germ tubes of *Candida albicans* show high specificity and sensitivity. Many authors also confirm the ability of the method in the diagnosis of systemic mycoses because this is used to distinguish for colonization with *C. albicans* in a systemic infection. This is very important for empiric therapy in patients (22).

Detection of galactomannan in serum is a feasible approach in adult neutropenic patients for the early diagnosis of invasive aspergillosis.

ELISA – Platelia tests perform higher sensitivity and they are more appropriate for the analysis of a great number of serum samples. On the other hand, LA tests are easier to perform in laboratory conditions (23). We must not forget also the costs of antigen-detection tests.

Real time PCR is currently one of the fastest diagnostic methods for fungal species, that are the most frequent causative agents of systemic infections (24; 25).

Molecular methods are faster and more accurate than the biochemical ones for the needs of diagnosis and identification of medically significant fungi. These methods are new, relatively inexpensive and certainly take less time to refine.

Further studies are needed on the usefulness of PCR in routine practice, as well as the

combination with other biomarkers as an optimal strategy. European Organisation for Research and Treatment of Cancer (EORTC) and Mycoses Study Group (MSG) includes PCR method for *Aspergillus* detection as a method for probable IFD, but PCR for *Candida* remains a research approach in most research centers, due to limited standardization (26).

## CONCLUSIONS

Genetic methods perform higher specificity than serological ones. They can significantly reduce detection time, but there are still no fully standardized protocols for isolating fungal DNA and performing PCR (27;28). Standardization is necessary for their introduction in the routine practice in mycological laboratories. Problems with the avoidance of contamination of clinical materials and with the development of methodologies to distinguish infection from contamination are to be resolved (29). Meta-analysis confirms that if both PCR and Elisa are consistently negative, then this is sufficient to rule out invasive aspergillosis (30).

Clinical mycology, unlike bacteriology, has a slower pace of development. The use of serological tests for antigens and antibodies, as well as the introduction of molecular techniques make non-cultural methods essential for the prediction of IFD. None of these tests, however, can be used as a stand-alone definitive diagnostic test. Compliance with clinical data, imaging studies, etc is essential (1).

Laboratory diagnosis of invasive fungal disease has to be used in conjunction with other diagnostic procedures such as culture technique, histological examination of biopsy samples, computed tomography (CT) imaging (31,32). Further research is needed to establish the benefits of PCR for fungal detection in routine practice (26).

## ACKNOWLEDGEMENT

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